ORIGINAL ARTICLE

A morphometric analysis of hepatitis B subviral particles shows no correlation of filament proportion and length with clinical stage and genotype

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Abstract

It was recently suggested that the composition of circulating hepatitis B subviral particles (SVPs) could be used to differentiate the various stages in chronic hepatitis B virus (HBV) infection, with significantly lower proportions of L and M proteins in inactive carriers than in individuals with chronic hepatitis. L protein is abundant in virions and filamentous SVPs but almost absent from spherical SVPs. We, therefore, performed a morphometric analysis of SVPs in these two groups of patients, by conducting a retrospective analysis on sera from 15 inactive carriers and 11 patients with chronic hepatitis infected with various HBV genotypes. Subviral particles were concentrated by centrifugation on a sucrose cushion, with monitoring by transmission electron microscopy. The percentage of filamentous SVPs and filament length for 100 SVPs was determined with a digital camera. The L protein PreS1 promoter was sequenced from viral genomes by the Sanger method. No marked differences were found between patients, some of whom had only spherical SVPs, whereas others had variable percentages of filamentous SVPs (up to 28%), of highly variable length. High filament percentages were not associated with a particular sequence of the L protein promoter, HBV genotype or even disease stage. High levels of circulating filamentous SVPs are probably more strongly related to individual host factors than to viral strain characteristics or disease stage.

KEYWORDS
electron microscopy, genotype, hepatitis B virus, liver disease stage, morphometric analysis, subviral particle

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1 | INTRODUCTION

Despite the availability of vaccination, hepatitis B virus (HBV) remains a major global public health threat, with about 300 million people chronically infected worldwide.[1] The clinical course of chronic infection, defined as the persistence of hepatitis B surface antigen (HBsAg) in serum, is characterized by several phases depending on host immune response efficiency and viral factors. In most individuals, the clinical course is silent, and liver complications are often diagnosed after decades of infection. Seroconversion for HBeAg is a key mechanism in this progression. Most HBeAg-negative patients are inactive carriers, with minimal HBV replication and no inflammation, but some go on to develop chronic hepatitis, with persistent viral replication and hepatic necro-inflammation. It is essential to differentiate between stable inactive carriers and patients with active disease or a risk of disease reactivation, because these two groups of patients are very different, particularly in terms of their risk of developing cirrhosis or hepatocellular carcinoma (HCC).[2,3] HBsAg seroconversion occurs in chronic HBV carriers, and this event defines the functional cure, but covalently closed circular HBV DNA (cccDNA) may persist for life in hepatocytes.

Viral and biochemical biomarkers have been evaluated, with a view to improving the monitoring of patients with chronic HBV infection. An ideal HBV biomarker would stratify patients by disease stage and risk of complications, for the prediction of functional cure, and the early evaluation of treatment response. The first markers to be validated were HBV DNA quantification by molecular biological methods and HBsAg quantification by immunoenzymatic assays.[4] High levels of these two biomarkers are strongly associated with poor prognosis and the occurrence of HCC, and both can be used as tools for characterizing the clinical phase of the patient, together with determinations of alanine aminotransferase (ALT) levels. New biomarkers, such as HBcrAg and HBV RNA levels, have recently been identified.[5] HBcrAg levels are well correlated with the amount of hepatic intracellular cccDNA, and automated assays are commercially available. The determination of HBcrAg levels can be used to predict the risk of fibrosis in HBeAg-negative patients. HBV RNA levels are predictive of HBsAg seroreversion, and a lack of HBV RNA detection could be used to distinguish between inactive carriers and patients with active disease. Moreover, these two new biomarkers accurately predict a sustained response during treatment monitoring and HBeAg seroconversion.

The composition of HBsAg has also recently been reported to differentiate the different stages of chronic HBV infection.[6,7] HBsAg consists of three proteins encoded by a single open reading frame in the HBV genome with three translation start codons. Depending on the site used for translation initiation, the large surface protein (L, containing the preS1, preS2 and S domains), the middle surface protein (M, containing the preS2 and S domains) or the small surface protein (S, containing only the S domain) is produced.[8] In one study, HBsAg-positive HBeAg-negative subjects in the inactive carrier

Significance statement

Hepatitis B virus is unique among viruses in being able to produce and secrete subviral particles (SVPs)—non-infectious viral particles devoid of capsid and genome—in much larger numbers than complete infectious Dane particles. These SVPs take two forms: spheres with a diameter of 22 nm and filaments of various lengths. We found that some patients had high levels of SVP filaments in their serum, whereas others had only the spherical form, and that the proportion of these forms was not correlated with liver disease stage or a specific viral genome sequence.

phase were found to have significantly lower proportions of L and M proteins than patients in the chronic hepatitis phase, and it was suggested that this marker was a better predictor than total HBsAg level in this population.[6] In another study, serum L (preS1) protein levels in HBeAg-negative or HBeAg-positive patients with chronic hepatitis B were found to be correlated with ALT level and were significantly higher in patients with higher grades of liver inflammatory activity on histological examination.[7] However, the proportions of the three surface proteins in the serum of infected subjects may be dependent on viral genotype, with the proportions of L and M relative to S being higher for genotypes B and D and lower for genotypes A, C and E.[9] These potential differences according to HBV genotype may complicate the interpretation of HBsAg composition in different clinical situations.

In addition to its location on virions (Dane particles), HBsAg circulates in the blood as the form of non-infectious subviral particles (SVPs). Some of these particles are spherical, with a diameter of 22 nm, whereas others form large filaments of various lengths, between 80 and 400 nm.[10] Each type of viral or subviral particle contains a different proportion of the three forms of HBsAg. The L protein is abundant in virions and filaments but almost absent from spheres, whereas all particles consist mostly of the S protein and, to a lesser extent, the M protein. In chronically infected subjects, SVPs circulate at levels 1000–100 000 times higher than virions; the L protein detected in serum therefore corresponds essentially to the L protein present in subviral filaments.[11] We hypothesized that the proportion of subviral filaments could be a relevant marker to differentiate between patients with chronic hepatitis and inactive carriers. In previous studies, the L protein was quantified in serum by specific ELISA and/or by a densitometric analysis of Western blots. Here, we used a well-characterized HBsAg-positive/HBeAg-negative population to monitor the precise proportions of the various circulating SVPs by electron microscopy and to determine the length of the filaments. Despite the limited number of patients, this morphometric SVP analysis showed that the proportion of filaments was not correlated to any particular stage of chronic infection or to infection with a particular HBV genotype.

2 | MATERIALS AND METHODS

2.1 | Patient samples

We performed a retrospective analysis of serum samples obtained from patients with chronic HBV infection followed at the hepatogastroenterology department of Tours University Hospital (France). We used samples that had been stored at −20°C in the serum bank of the hospital's virology laboratory (Table 1). The principal inclusion criterion was chronic HBV infection, as demonstrated biologically by positivity for HBsAg for at least 6 months. The inclusion criteria for inactive carriers (n = 15) were negative results for the detection of HBeAg and positive results for the detection of anti-HBe antibody (Architect and Alinity i HBe Ag and Ab assays, Abbott)), an estimated METAVIR stages F0-F1 determined by blood test (Fibrometer®) or liver stiffness measurement (Fibroscan®), associated with an absence of liver cell injury characterized by a normal alanine aminotransferase level (ALT ≤2.0-fold ULN).

Inclusion in the chronic hepatitis group (n = 11) was based on an estimated METAVIR score of F3-F4 for the same non-invasive approaches, and HBeAg-negative status (HBeAg-positive patients were excluded to make it possible to compare this population directly with HBeAg-negative inactive carriers), with near-normal-to-high ALT levels. The principal exclusion criteria were as follows: current or planned antiviral therapy for HBV infection, coinfection with HCV or HIV, malignant comorbidities, hepatocellular carcinoma or hepatic metastases. For six patients, we obtained a second sample collected about 6 months after the first. This study was performed after patient information and anonymization of the data in accordance with French Reference Methodology MR-003 and the Declaration of Helsinki. Data collection was approved by the *Commission Nationale de l'Informatique et des Libertés* (no. 2019_022), and sample collection was approved by the *Ministère*

de l'Enseignement Supérieur et de la Recherche (authorization no. 2016_094).

2.2 | Standard laboratory assessment

Total HBsAg levels were quantified with the Alpha® ELISA Kit 4110 for HBsAg. HBV viral loads were quantified using the Abbott Real-Time HBV assay on an Abbott M2000 Real-Time system. Genotyping was performed by amplifying and Sanger sequencing the Pol/RT domain (aa rt1-rt344), as previously described.[12] Briefly, HBV DNA was extracted with the DSP Virus kit (Qiagen) on an EZ1 Advanced XL system. Pol/RT domains were amplified as previously described,[17] sequenced with BigDye Terminator Mix 1.1 (Applied Biosystems) and analysed with CLC Genomics Workbench software V3 (CLC Bio). Genotype was determined with two online algorithms: Geno2pheno [HBV] v2.0 (Max Planck Institute) and HBV tool v0.8, based on Stanford HIValg-Software (HIV-GRADE).[13,14]

2.3 | Sucrose cushion enrichment

For the analysis of subviral particles from patient samples, 500 μL of serum was layered on a 300 μL 10% sucrose cushion (wt/vol, in PBS) and subjected to ultracentrifugation at 200 000 x g and 4°C for 2.5 h (Sorvall WX 90 Ultra-superspeed, rotor Fiberlite™ F50L-24), and the pellet was resuspended in 40 μl phosphate-buffered saline (PBS).

2.4 | Electron microscopy

Formvar/carbon-coated nickel grids were deposited on a 10 μL drop of concentrated serum for 3 min and rinsed three times with

TABLE 1 Summary of patient characteristics

	Inactive carriers n = 15	Chronic hepatitis n = 11
Age (mean ± SD)	30.4 ± 11.5 (15–51)	48.9 ± 25.4 (18–86)
Sex (F:M)	0.6	0.2
HBV genotype		
A	2	4
B	1	1
C	0	0
D	3	1
E	8	4
ND	1	1
ALT (IU/L)	18.3 ± 7.9 (11.0–38.0)	49.5 ± 36.3 (14.0–136.0)
HBsAg ($\log_{10}$ IU/mL)	3.2 ± 0.3 (2.7–3.6)	3.0 ± 0.5 (1.9–3.6)
HBV DNA ($\log_{10}$ IU/mL)	2.9 ± 0.9 (1.7–4.6)	3.3 ± 1.6 (2.0–6.6)

Note: For two patients, it was not possible to determine viral genotype (ND).

PBS. The grids were washed in distilled water, and negative straining was performed with three consecutive contrast steps with 2% uranyl acetate (Agar Scientific, Stansted, UK), before observation under a transmission electron microscope (JEOL JEM-1400 Plus, Tokyo, Japan). Subviral particles (spheres and filaments) were visually identified and counted during observation, with a sufficient number of fields analysed for the evaluation of 100 consecutive subviral particles in total. Electron micrographs of these fields were recorded with a digital camera driven by Digital Micrograph software (GMS 3, Gatan, Pleasanton, CA, USA). Filaments were measured on these electron micrographs, with Gatan Digital Micrograph software.

2.5 | PreS1 promoter sequencing and analysis

Before sequencing, viral DNA was extracted and purified from serum with the EZ1 Qiagen® instrument. Nested PCR was performed, to amplify the HBs gene and its upstream promoter. For the first amplification, we used the Q5SMD-8-32-2017-F and RTr primers (Table S1), and, for the second, we used the A1-F and 786r primers. Sanger sequencing of the PreS1 promoter region was performed on the DNA products, with the A1F, 786r, OS1 and Pol 1 M primers, on an Applied Biosystems® AB Prism 3130 XL. Evolutionary history was inferred by the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is indicated above the branches. The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were calculated by the number of differences method and are expressed as the number of base differences per sequence. All ambiguous positions were removed for each sequence pair (pairwise deletion option). In total, 65 positions were included in the final dataset. Evolutionary analyses were conducted in MEGA X.

2.6 | Statistical analysis

Statistical analysis was performed with Wilcoxon–Mann–Whitney tests in GraphPad Prism 5 software (GraphPad Software Inc. La Jolla, CA).

3 | RESULTS

3.1 | Subviral particle characteristics

Electron microscopy analysis was performed on serum samples, to count spherical and filamentous SVPs and determine their proportions, and to measure filament lengths (Figure 1). During these observations, Dane particles were encountered only rarely and were not counted. This analysis highlighted marked differences between patients, some of whom had only spherical SVPs, whereas others had variable percentages of filamentous SVPs (up to 28%) of various lengths (Table 2).

3.2 | Percent and mean length of filaments in inactive carriers and patients with chronic hepatitis and as a function of HBV genotype

The percent filaments did not differ significantly between inactive carriers and patients with chronic hepatitis (Figure 2A). No significant differences in the length of the filaments were observed between individuals with filamentous SVPs in their serum (Figure 2B). No particular HBV genotype was specifically associated with a higher percentage of filaments or with longer filaments (Table 3). An analysis of the percentage and length of filaments present in the serum of patients, as a function of serum ALT level also revealed no particular correlation (Figure 3A and B). Finally, we investigated whether the presence of filaments was a stable phenomenon, by analysing serum samples collected 6 months after the first sample, when available. We found that the percentage of filaments remained stable over time, displaying little or no change in these patients (Figure S1).

3.3 | PreS1 promoter sequencing

Sanger sequencing of the HBs promoter region revealed four conserved domains, corresponding to binding sites for the HNF1, HNF3 and Sp1 transcription factors, and a TATA-box (Figure S2A). Five variable positions have been identified leading to thymidine-to-cytosine substitutions (T2739C, T2754C, T2757C, T2772C, T2775C). No mutations have been specifically associated with a higher percentage of filaments. Phylogenetic analysis by the neighbour-joining method did not reveal any close relationships between sequences from patients with filamentous SVPs (Figure S2B).

4 | DISCUSSION

Using specific anti-preS1 monoclonal antibodies, several groups have recently developed ELISAs for specific quantification of the L protein in the serum of chronic HBsAg carriers, and for comparisons of its level with the total amount of HBsAg.[6,7,9,15,16] These semi-routine assays have made it possible to study a large number of serum samples, and, for a few selected sera, a good correlation with the presence of the L protein on Western blotting has been reported.[6,9] One study showed that, in a population of HBeAg-negative subjects, the proportion of L can be used as a biomarker for differentiating between inactive carriers and those developing chronic active hepatitis B. For total HBsAg levels above 1000 IU/mL, the proportion of L was significantly lower in inactive carriers than in patients with chronic active hepatitis B.[6] More recently, it was found that, in HBeAg-positive patients treated with pegylated

FIGURE 1 Electron microscopy photographs representative of the observations made on the patients' sera. (A) and (B) Serum from patient IC4 containing almost exclusively spherical subviral particles (white arrowheads). (C) and (D) Serum from patient CH2 presenting many filaments of various lengths (black arrowheads) and spherical particles (white arrowheads). In such patients, filaments were measured to determine their mean size, as shown for one filament in each of these two photographs. IC: Inactive carriers. CH: Chronic hepatitis
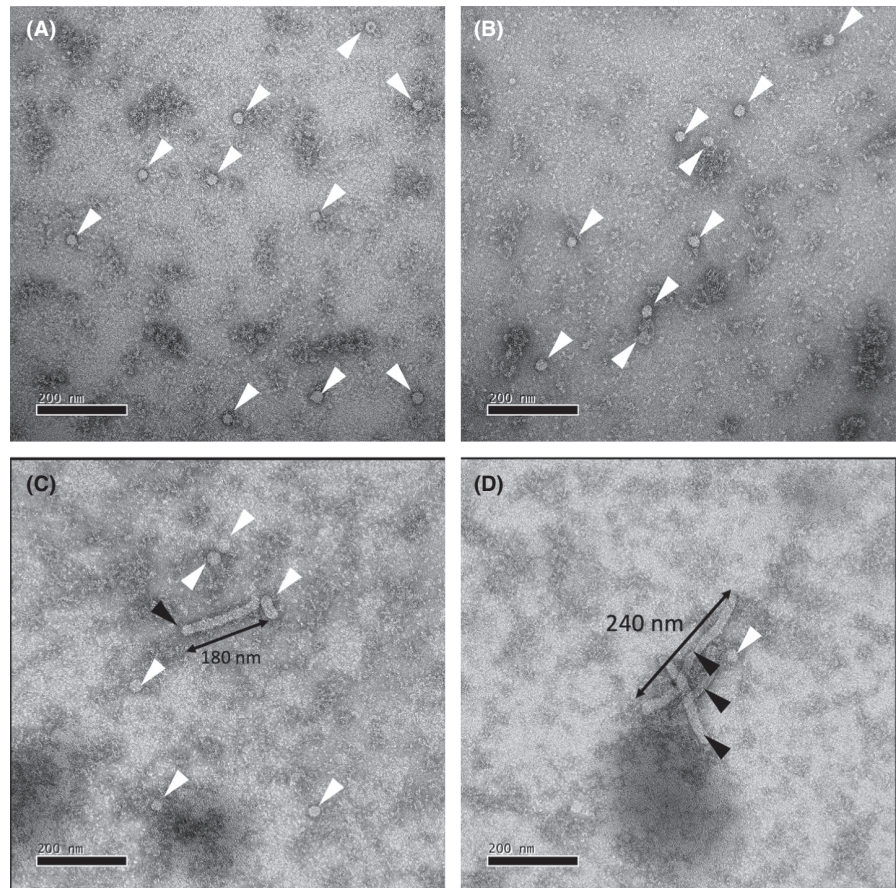
interferon (peginterferon) alfa-2a, L protein levels decrease before total HBsAg levels and before HBsAg loss; these findings suggest that the L/HBsAg ratio is a potentially interesting biomarker to predict HBV cure in treated patients.[16] However, another study concluded that L quantification has no advantage over the established quantitative assay for HBsAg for predicting the response to peginterferon alfa-2a therapy in HBeAg-positive patients.[15] However, these discrepancies may reflect the different proportions of the HBV genotypes in the study populations, as the proportions of circulating L, M and S proteins have been reported to be genotype-specific.[9] A potential association of the serum L protein levels with severity of infection has also been studied. L protein levels were found to be significantly correlated with ALT level and a higher grade of liver inflammatory activity.[7] The L/HBsAg ratio was found to reflect liver fibrosis stage more directly than total HBsAg level alone.[7]

The L protein in serum is strongly associated with filamentous SVPs. We therefore investigated whether the number and/or length of circulating filaments was related to chronic active liver disease or liver disease severity. This hypothesis was also supported by the observation of intracellular long SVP filament accumulation in a transgenic mouse model of L protein overexpression displaying significant cytotoxicity.[17] Direct cell toxicity due to the accumulation of filamentous SVPs has also been suggested in bioclinical studies of chronic HBV carriers.[18] The death of injured hepatocytes may be responsible for the release of these filamentous SVPs into the bloodstream.

Investigations in cellular models have shown that spherical and filamentous SVPs seem to follow different morphogenesis and secretory pathways. S-HBs initially self-assemble into filamentous SVPs in the lumen of the endoplasmic reticulum (ER), before transportation to the ERGIC (a compartment intermediate between the ER and the Golgi apparatus), where they are converted into spheres. Spherical SVPs are then released from the cell via the constitutive secretory pathway.[10] Recent data have shown that filamentous SVPs with high L-HBs content are less efficiently converted into spheres, and are, thus, excluded from the classic secretory pathways, due to their size. These filaments may be released in multivesicular bodies, such as infectious Dane particles,[19] but they undoubtedly also accumulate in cells. For all these reasons, the filaments present in the serum of patients may reflect the cell injury caused by their accumulation in the liver and constitute a potential biomarker of pathogenicity. However, our analysis shows that neither the percentage of filamentous SVPs nor their length is correlated with the clinical status of the patient or the intensity of hepatic cytolysis assessed by determinations of ALT level.

Our analysis revealed no marked differences between genotypes. Despite the small number of patients included, our results for the quantification of HBsAg levels are consistent with those of Kuhnhenn et al., who reported significantly lower qHBsAg levels for genotype B than for genotypes A, D and E.[20] However, we found no evidence for a difference in the percentage of filaments between genotypes. This finding contrasts with those of studies

TABLE 2 Characteristics of the subviral HBV particles circulating in inactive carriers and patients with chronic hepatitis

	Genotype	HBsAg (log$_{10}$ IU/ mL)	Filament %	Filament length in nm (mean ± SD)	Cumulative filament length in nm
Inactive carriers (IC)					
1	A	2.7	1	NA	NA
2	B	3.1	22	133 ± 67	2917
3	E	3.6	4	54 ± 10	215
4	E	2.7	1	NA	NA
5	D	3.4	6	68 ± 12	410
6	E	3.5	14	89 ± 41	715
7	D	3.4	7	81 ± 33	565
8	D	3.3	8	82 ± 22	655
9	E	3.2	4	110 ± 18	440
10	E	3.4	1	NA	NA
11	E	3.2	0	NA	NA
12	E	2.9	24	163 ± 60	2948
13	E	3.6	1	NA	NA
14	ND	2.8	1	NA	NA
15	A	3.0	3	162 ± 36	648
Chronic hepatitis (CH)					
1	D	3.3	6	68 ± 28	410
2	E	3.6	28	103 ± 49	2886
3	A	3.5	1	NA	NA
4	B	1.9	0	NA	NA
5	A	3.5	0	NA	NA
6	ND	2.3	0	NA	NA
7	A	3.2	8	110 ± 20	440
8	E	2.8	6	120 ± 25	482
9	E	3.1	8	185 ± 45	1483
10	E	2.7	14	140 ± 50	2095
11	A	3.1	0	NA	NA

Note: Cumulative filament length is the sum of the lengths of all the filaments identified.
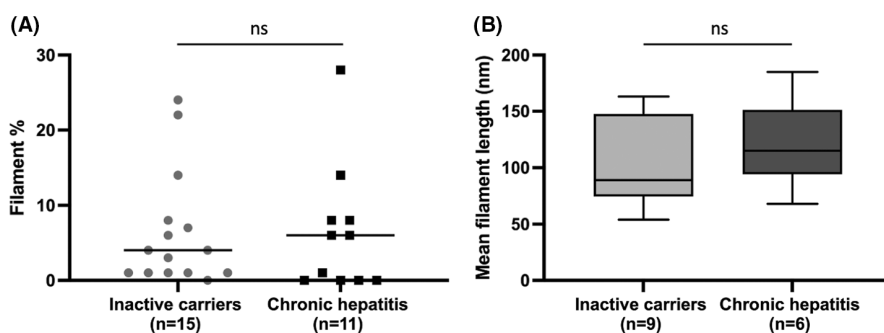

FIGURE 2 Characteristics of the subviral HBV particles circulating in inactive carriers and in patients suffering from chronic hepatitis. (A) Percentage of SVPs in the filamentous form; (B) mean filament length in nanometres (nm) for patients with circulating filamentous SVPs

reporting differences in the amount of circulating L protein, which has been shown to be greater for genotypes B and D than for genotypes A, C and E.[9] However, the methods of L quantification used in these studies were not directly comparable with ours. The previous studies were based on ELISA with the immunocapture of SVPs by anti-preS1 antibodies and detection with anti-S antibodies. This approach may underestimate the amount of L in long filaments and overestimate the amount of L in very short filaments. Our study based on a visual identification and counting of SVPs by electron microscopy is original in that it addresses both the relative number of filamentous SVPs and their length, which varied considerably between patients.

TABLE 3 Characteristics of the subviral HBV particles for all patients as a function of HBV genotype

HBV genotype	n	HBsAg (log$_{10}$IU/mL)	Filament % (mean ± SD)	Filament length in nm (mean ± SD)
A	6	3.2 ± 0.3	2.2 ± 3.1	64 ± 63
B	2	2.5 ± 0.8	11 ± 15.6	66 ± 94
C	None	-	-	-
D	4	3.4 ± 0.1	6.8 ± 1.0	75 ± 8
E	12	3.2 ± 0.4	8.8 ± 9.4	110 ± 50

Note: For each genotype, mean filament length was calculated taking only patients with at least three filaments in their serum samples into account.

FIGURE 3 Absence of correlation between hepatic cytolysis (serum ALT levels) and the percent filamentous SVPs (A), or filament length (B), for all circulating SVPs, in all patients
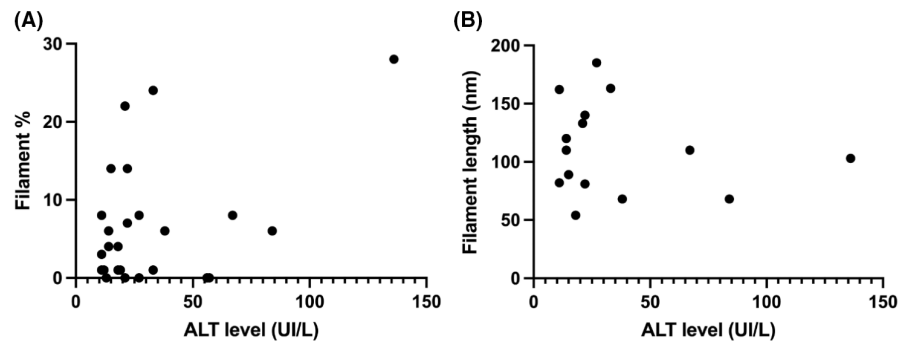
Thus, our study shows that the proportion of filamentous SVPs is extremely variable between patients and is not correlated with HBV genotype, preS1 promoter mutations or liver disease stage. Furthermore, we show here that, at least in a small number of patients, the proportion of filamentous SVPs remains stable over time. The reasons for these differences between patients remain unclear but may reflect the translation of L-HBs from a 2.4 kb preS1 mRNA, independently of the translation of M-HBs and S-HBs, which are produced from a separate 2.1 kb mRNA. Differences in filamentous SVP production may be due to host transcription factors upregulating preS1 mRNA synthesis, increasing L-HBs production and, thus, filament formation.[21]

In conclusion, this study is the first to monitor HBV subviral particle profiles with precision in patients. Our results require, however, confirmation with larger cohorts. Also, it will be important in further investigations to correlate the amount of filaments with preS1/L-specific Western bot analysis and ELISA, which was not possible in this retrospective study due to the lack of remaining serum for most of the studied patients. Nevertheless, at this stage, our study suggests that the presence of HBV filaments is not associated with disease stage or viral genotype.

AUTHOR CONTRIBUTIONS

Sébastien Eymieux, Emmanuelle blanchard and Philippe Roingeard conceptualized and designed the experiments, analysed the data and wrote the manuscript. Sébastien Eymieux, Christophe Hourioux, Alain Moreau and Romuald Patient performed the experiments. Sébastien Eymieux, Julien Marlet and Catherine Gaudy-Graffin were involved in the routine biological analyses and patient selection. Louis d'Alteroche performed clinical examinations. All authors have reviewed and approved the final manuscript.

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